Process for preparing of full-function helper virus used for producing recombinant adeno-associated virus and uses thereof

The present invention relates to the field of genetic engineering in virus, particularly to the replicating and packaging function system that are necessary for large production of recombinant adeno-associated virus (rAAV). The invention relates to a method of producing recombinant herpes simplex virus (HSV1-rc) that is loaded with rep-cap genes (403kb) of type2 adeno-associated virus (AAV-2) and the use thereof in the production of recombinant adeno-associated virus. The recombinant herpes simplex virus can provide the complete helper functions that are necessary for rAAV plasmid to be replicated and packaged in cells to be rAAV virus particles, and can be used in large preparation of rAAV. The production of HSV1-rc is carried out based on the modification for a set of cosmids (Set C cosmid, including cos6, cos14, cos28, cos48, cos56) that contain the full-length genome of rAAV virus particle. Lots of infectious rAAV virus particles can be produced by using HSV1–rc to infect the cells transfected with rAAV vector plasmid or the cell strain that carry stably rAAV vector plasmid. Exogenous genes can be introduced into mammalian cells for expression by using rAAV thus produced.

Adeno-associated virus is a member of parvovirus, with a single-stranded DNA genome consists of 4682nt. AAV is a dependent virus of which the replication needs the participation of other viruses such as adenovirus or herpes simplex virus, or other helper factors. The genome of AAV will be integrated into the chromosome of cells in latency upon AAV infection in the absence of helper virus, without generating progeny viruses.

The full-length genome of AAV-2 has been cloned into the plasmid of E. coli. Two 145bp length of inverted terminal repeat sequences (ITR) are contained in the genome. The two ITRs are replication origins of AAV genome and participate in such functions as replication, integration, or package of AAV, etc.. The rest parts of the genome are divided into two functional regions, rep gene region and cap gene region. The rep gene encodes four different types of products: Rep78, Rep68, Rep52, Rep40, which are regulatory proteins necessary for the replication and gene expression of AAV. The cap gene encodes three types of structural proteins: VP1, VP2, VP3 which are assembled together into the capsid of AAV . The proteins encoded by rep and cap genes are trans-acting proteins in the toxicogenic replication of AAV.

AAV is considered as one of the ideal candidate vectors in gene therapy. A rAAV virus into which exogenous genes can be transduced has been constructed in a lot of laboratories. The main structural characteristic of AAV vector plasmid is the remove of rep-cap genes from the genome of the virus and the substitution of the desired DNA fragment.

The classical method of producing rAAV is transducing rAAV vector plasmid and a helper vector that contains rep-cap genes into the cells that has been infected by adenovirus or herpes simplex virus. 2-3 days later, rAAV as well as adenoviruses and herpes simplex viruses, can be harvested from the supernatant and the pathological cells. Adenoviruses and herpes simplex viruses can be inactivated by

heat treatment(at 55°C for 30min to 2hr) without affecting the activities of AAV.

Although the method of producing rAAV is simple, it still exists a lot of disadvantages. Firstly, cells need to be transfected in each preparation of rAAV. One of the factors that result in lower titer of rAAV is the low efficiency of transfection and co-transfection due to the restriction of the transfection method itself. Furthermore, it is difficult for cells to be transduced on a large scale by transfection method by now, accordingly it can't meet the needs of producing rAAV largely. Thus a system and method that can be used to produce rAAV largely needs to be studied.

Yan ziying et al have ever filed a patent application named "A herpes simplex virus vector that can be used to pack adeno-associated virus and the use thereof" (Chinese patent application number 96120549.0, publication number CN1159480A) in 1996. The application described that the rep-cap genes of AAV-2 are located in the amplicon vector plasmid of HSV1 to construct pHSV–AAV (+/-). The plasmid was introduced into cells, and a mixed virus of a wild-type HSV-1 and a pseudovirus that contained rep-cap genes were obtained in the presence of the wild-type HSV-1, wherein the mixed virus can provide the complete helper function for the replication and package of rAAV. Recently, Conway et al (Conway JE et al, Recombinant adeno-associated virus type 2 replication and packaging is entirely supported by a herpes simplex virus type 1 amplicon expressing rep and cap J. Virol. 71(11): 8780-8789, 1997) also reported the similar studies. However the pseudovirus in the mixed virus occupied a small proportion (<10%), and provided limited helper function; furthermore the proportion between the pseudovirus and the wild-type virus is unstable in virus passage, thus unfit for quality control in large production.

The purpose of the invention is to provide a technical method that is used to produce rAAV conveniently and largely and a full-function helper virus HSV-rc. The purpose of the invention is carried out by providing recombinant cosmid that contain rep-cap genes and the construction method thereof, HSV-rc and the construction method thereof as well as the method of producing rAAV by using HSV-rc.

The full-function helper virus HSV-rc presented in the invention is a recombinant HSV-1 virus with the characteristic of a copy of rep-cap genes (4.3KB, the direction is not restricted) being inserted into the genome of HSV-1. In the two HSV-rc virus constructed by the invention, rep-cap genes are inserted respectively into the site Xbalin UL2 gene (encodes uracil-DNA glycosylase) and UL44 gene(encodes glycoprotein C) of HSV-1, and the obtained recombinant viruses are named respectively HSV-rc/ΔUL2 (figure 1a) and HSV-rc/ΔUL4 (figure 1b). The products encoded by UL2 and UL44 genes are not necessary for the proliferation and passage of HSV-1 in in-vitro cell culture. Both of the two recombinant HSV1 viruses may be proliferated and subcultured stably in HSV sensitive cells (e.g., BHK-21).

The HSV-rc virus generated by the invention is produced based on the modification for a set of cosmids (Set C cosmid, including cos6, cos14, cos28, cos48, cos56) (Conningham, C Davision AJ. A cosmid-based system for constructing mutants of herpes simplex virus type 1. Virology, 1993, 197:116-124) that contain the full-length genome of rAAV virus particle. Firstly, the rep-cap genes of AAV-2 are inserted into the genome of HSV-1 contained in one of the cosmids by rcombinant DNA technology.

Then the recombinant cosmid that has been inserted rep-cap genes is cut with the other four corresponding cosmids together by enzyme to remove the backbone part of the cosmids before being co-transfected into HSV1 sensitive cells such as BHK-21 using liposome method. Recombinant virus is generated through homologous recombination of the five fragments of HSV-1.

The cells transfected with rAAV vector plasmid that contains reporter gene GFP (Green fluorescence protein) are infected by the recombinant HSV1 virus, and the obtained supernatant of the cell lysate is used in infecting the cultured mammalian cells, a lot of green cells can be observed under fluorescent microscope (the wavelength eradiated is 490nm). The result demonstrates that the rAAV viruses generated are infectious, by which exogenous genes can be introduced into cells for expression.

The recombinant plasmid pEBUF5 that contains GFP gene is constructed based on the plasmid pBDZ(+) (Chinese patent application 97116981.0) in the invention. The plasmid is introduced into 293c18 cells (ATCC CRL10852, F9766, the gene EBNA1 of EBV is contained and expressed therein), and the resistant cell strain HygromycinB thus obtained is named 293c18/EBUF5. A lot of infectious rAAV/GFP virus particles may be produced conveniently by using HSV1-rc virus to infect the cell strain 293c18/EBUF5 that carries stably the rAAV vector plasmid pEBUF5, thus a large-scale production of rAAV can be carried out by this method.

The original biological materials that are used in the invention to construct recombinant HSV-rc are:

Set C cosmids: consist of five cosmids that carry respectively the full-length genome of HSV-1: cos6, cos14, cos28, cos48, cos56 (Conningham C, Davision AJ. A cosmid-based system for constructing mutants of herpes simplex virus type 1. Virology, 1993, 197:116-124), and is kindly presented by Davision AJ. There are terminal repeat sequences between each terminal HSV-1 fragment loaded in one cosmid and that in another cosmid, which is the basis of homologous recombination for the five genome fragment of HSV-1 in cells.

pSub201: Samulski et al, A recombinant plasmid from which an infectious adeno-associated virus genome can be excited in vitro and its use to study viral replication.

The production method of recombinant virus HSV-rc

The same strategy and method as producing recombinant virus HSV1-lacZ100 (Wu, XiaoBing et al, Chinese patent application number 98101753.3) are used.

In the HSV-1 genome fragments that are loaded in cos6 and cos56 cosmids there is respectively one single enzyme cleavage site XbaI that are located respectively in UL2 and UL44 genes. Rep-cap genes are removed from pSub201 by cutting with XbaI, and inserted into the site XbaI in cos6 and cos56 to construct recombinant cosmids cos6-rcΔUL2 (figure 2a) and cos6-rcΔUL44(figure 2b). The two recombinant cosmids are preserved in the strain of E. coli DH5α (MAX Efficiancy DH5α, GIBCO#18258-012). The strains that respectively contain the two recombinant cosmids have been

deposited in China General Mcirobiological Culture Collection Center on September 24, 1998. The accession numbers are respectively CGMCC No.0361-1 and CGMCC No.0361-2.

cos6-rcΔUL2 and cos14, cos28,cos48,cos56 together is called Set H (figure 2a), and cos56- rc ΔUL44 and cos6, cos14, cos28, cos48 together is called Set I (figure 2b). The five cosmids of Set H or Set I are mixed in equal mol, cut by PacI to remove the backbone of cos, and co-transfected into BHK-21 cells by liposome, homologous recombination occurs among the five HSV-1 fragments in cells and the recombinant virus HSV-rc is generated: the cells appear pathological 5 days later, and the cultured supernatant is collected after complete pathological, and centrifuged at 2000r/min for 5min, and the obtained supernatant is sotred at –20℃. The recombinant HSV-rc produced by Set H is named HSV1-rcΔUL2 (figure 1a), and the recombinant HSV-rc produced by Set I is named HSV1-rcΔUL44 (figure 1b). The probability of producing recombinant HSV-1 that contains target DNA fragment through the method is 50-100%. Pure recombinant virus can be easily obtained through plaque screen.

The preparation of the full-function helper virus used for the producing rAAV and the use thereof of the invention are specified in the following examples, which should not be construed as limitations for the contents of the invention.

EXAMPLE 1 the preparation of cosmid DNA

Cosmid DNA were extracted by the method of alkaline lysis according to Molecular Cloning –A Laboratory Manual, 2nd edition (Sambrook J. et al, 1986), and purified by the method of polyethylene glycol precipitation.

EXAMPLE 2 the preparation of recombinant HSV-rc

The five cosmids of Set H or Set I were mixed respectively in equal mol, and cut by PacI to remove the backbone of cos (separation removal was not necessary), and extracted respectively one time by phenol, phenol/chloroform (1:1), the supernatant was removed, and the DNA therein was precipitated with 2.5 times absolute ethanol. 20ul Lipofactamine (GIBCO BRL) and 10ug DNA were co-transfected according to the product description into 80% confluenced BHK-21 cells (about 2×10^6 cells), homologous recombination would occur among the five HSV-1 fragments in cells and the recombinant virus HSV-rc would be generated. The cells were cultured at 37□ in 1640 medium supplemented with 2% FBS after transfecting for 24h, and the medium was changed one time per day. The cells appeared pathological 5 days later, and the cultured supernatant was collected after complete pathological, and centrifuged at 2000r/min for 5min, and the obtained supernatant is stored at –20℃. The recombinant HSV-rc produced by Set H is named HSV1-rcΔUL2, and the recombinant HSV-rc produced by Set I is named HSV1-rcΔUL44. Pure HSV1-rcΔUL2 and HSV1-rcΔUL44 could be obtained after two times plaque purity of the recombinant viruses.

EXAMPLE 3 the construction of cell strain 293c18/pEBUF5

The recombinant plasmid pEBUF5 that contained GFP gene was constructed based on the plasmid pBDZ(+) (Chinese patent

application 97116981.0), the structure of pEBUF5 was shown in figure 3. The plasmid pEBUF5 was introduced into 293c18 cells (ATCC CRL 10852, F9766) by the method of liposome, and the cells were cultured with 200ug/ml Hygromycin B selection for 10-15d, the obtained resistant cell strain was named 293c18/EBUF5.

EXAMPLE 4 the cells having been transfected with pAAV-GFP were infected by HSV-rc to prepare rAAV-GFP

The cells transfected with rAAV vector plasmid that contained reporter gene GFP (Green fluorescence Protein gene) were infected by recombinant HSV1, the transfected cells were froze and thawed for 4 cycles to release rAAV-GFP after pathological, the cell debris were removed by low centrifugation, and the supernatant was inactivated at 56℃ for 30min for use in infecting cultured mammalian cells.

EXAMPLE 6 the cell strain 293c18/pEBUF15 was infected by HSV-rc to prepare rAAV-GFP

The cell strain 293c18/pEBUF15 that carried rAAV vector plasmid pEBUF5 stably was infected by 0.5-5moi HSV1-rc, the cells appeared complete pathological 24-28h later, the cells and the medium thereof were froze and thawed for 4 cycles, and centrifuged at 1000r/min for 5min, a lot of rAAV-GFP virus were contained in the supernatant. Lots of infectious rAAV/GFP virus particles may be produced conveniently by this method, thus large-scale production of rAAV could be carried out.

EXAMPLE 7 rAAV viruses were transduced into cultured cells

1ml supernatant of rAAV-GFP viruses were added into the cultured BHK cells (with 80% confluence), a lot of green cells could be observed under fluorescent microscope (the wavelength eradiated is 490nm) 24-28h later. The result demonstrated that the generated rAAV viruses were infectious, by which exogenous genes could be introduced into cells for expression.

Figure 1a illustration of genome structure of recombinant HSV1-rc/ΔUL2
Figure 1b illustration of genome structure of recombinant HSV1-rc/ΔUL44
Figure 2a structure of cos6-rc/ΔUL2 and the cosmid Set H combinations
Figure 2b structure of cos56-rc/ΔUL44 and the cosmid Set I combinations
Figure 3 structural illustration of pEBUF5

What we claimed is:

The present invention relates to the field of genetic engineering in virus, particularly to the replicating and packaging function system that are necessary for large production of recombinant

adeno-associated virus (rAAV). The invention relates to a method of producing recombinant herpes simplex virus (HSV1-rc) that is loaded with rep-cap genes (4.3kb) of type 2 adeno-associated virus (AAV-2) and the use thereof in the production of recombinant adeno-associated virus.

1. A strategy of the invention for preparing a helper virus HSV-rc that can provide the full-function needed for the replication and package of rAAV, with the characteristics that, gene manipulation is done on a set of cosmids (Set C) that contain the full-length genome of HSV-1, rep-cap genes are inserted into the genome fragment of HSV-1, the resulting cosmid is co-transfected with the other four cosmids into cells, and the recombinant HSV-1 that contains rep-cap genes is obtained. The recombinant virus can provide both helper virus function needed for producing rAAV and the function of rep-cap genes, and thus is a full-function helper virus for producing rAAV.

2. Two HSV-rc constructed in the invention with that characteristics that, rep-cap genes are inserted respectively into the Xbal site in UL2 gene (encodes uracil-DNA glycosylase) and UL44 genes (encodes glycoprotein C) of HSV-1, and the obtained recombinant viruses are called respectively HSV-rc/ΔUL2 and HSV-rc/ΔUL4.

3. Process for inserting rep-cap genes into the HSV-1 genome fragments loaded in cosmids. Taking advantage of the characteristic of one single enzyme cleavage site Xbal (locates respectively in UL2 and UL44 genes) existing respectively in the HSV-1genome fragment loaded in cos6 and cos56 cosmids, rep-cap genes are removed from pSub201 by cutting with Xbal, and inserted into the site Xbal in cos6 and cos56 to construct recombinant cosmids cos6-rcΔUL2 and cos6-rcΔUL44. The two recombinant cosmids are preserved in the strain of E. coli DH5α (MAX Efficiency DH5α, GIBCO#18258-012). The strains that respectively contain the two recombinant cosmids have been deposited in China General Mcirobiological Culture Collection Center on 24th September 1998. The accession numbers are respectively CGMCC No.0361-1 and CGMCC No.0361-2.

4. The strategy, HSV-rc, or process according to claims 1, 2, or 3, wherein rep-cap genes are inserted into the other parts of the genome of HSV-1 through gene manipulation on a set of cosmids that contain full-length genome of HSV-1, and the recombinant HSV-rc with different inserting positions are produced.

5. The strategy, HSV-rc, or process according to claims 1, 2, or 3, wherein the recombinant HSV-rc with rep-cap genes being inserted into more than two positions.

6. The method of producing rAAV by infecting the cell strain that contains rAAV vector element using the recombinant virus HSV-rc according to the present invention.

ABSTRACTS

The present invention includes a method of producing recombinant herpes simplex virus (HSV1-rc) that is loaded with rep-cap genes of type 2 adeno-associated virus (AAV-2) and the usage thereof in the production of recombinant adeno-associated virus (rAAV). The recombinant herpes simplex virus can provide the complete helper functions that are necessary for rAAV plasmid to be replicated and packaged to be rAAV virus particles, and can be used in large preparation of rAAV. The production of HSV1-rc is carried out based on the modification for a set of cosmids (Set C cosmid, including cos6, cos14, cos28, cos48, cos56) that contain the full-length genome of rAAV virus particle. Firstly the rep-cap genes of AAV-2 are inserted into the genome of HSV-1 in one of the cosmids by recombinant DNA technology, for example inserted into the HSV1UL2 gene in cosmid 6 to construct cos6-rcΔUL2, or inserted into the HSV1UL44 gene in cosmid 56 to construct cos56-rcΔUL2. Then the recombinant cosmid that has been inserted rep-cap genes is cut with the other four corresponding cosmids together by enzyme to remove the backbone part of the cosmids, and transfected into HSV1 sensitive cells such as BHK-21 by the method of liposome. HSV1–rc is generated through homologous recombination among the five fragments of HSV-1 in the cells. Lots of infectious rAAV virus particles can be produced by using HSV1–rc to infect the cells transfected with rAAV vector plasmid or the cell strain that carry stably rAAV vector plasmid. Exogenous genes can be introduced into mammalian cells for expression by using rAAV thus produced.

Construction of a series of universal adeno-associated virus vectors and uses thereof

The present invention relates to the field of biotechnology.

Gene therapy, developed since 1990s, a brand new model for treating diseases, introduces therapeutic genes into human body to exert therapeutic effects. At present, more than 300 clinical solutions for gene therapy have been approved all over the world, and hundreds of thousands people have received such therapy. And the subjects for gene therapy have extended from the original genetic diseases and tumor into cardio-vascular diseases, infectious diseases, and so on.

The introduction of therapeutic genes into the cells of human body is the indispensable step, while suitable vectors are needed for the delivery of therapeutic genes. There are two kinds of vectors useful for the gene therapy, namely viral vectors and non-viral vectors. Among others, type 2 adeno-associated virus vector, a tiny virus defective in replication, is promising of being an ideal gene therapy vector due to its non-pathologic nature, abilities to infect cells post mitosis and integrate site-directedly into human chromosome 19, and so on, and thus are highly regarded in recent years in gene therapy researches. The conventional methods for preparing a recombinant adeno-associated virus (rAAV) carrying exogenous genes involve two plasmids: one is a vector plasmid carrying exogenous genes (i.e., therapeutic genes) expression cassette and AAV-2 inverted terminal repeats (ITR), in which ITR are the shortest cis-acting sequence; the other is a helper plasmid containing AAV-2 rep and cap genes, which provides trans-proteins necessary for the replication and package of rAAV. Co-transfection of these two plamids into the cell, followed by infection with helper virus (such as adenovirus or herpes simplex virus), would package a rAAV pseudovirus particle containing the exogenous genes. We have invented a full-function helper virus for large scale production of rAAV (CN Appl. No. 98120033.8), which simplified the production steps and increased the yields.

The first step for preparation of rAAV needs the construction of a AAV vector plasmid carrying the therapeutic genes. At present, a conventional method for construction of a AAV vector plasmid starts from a plasmid comprising the whole genome of AAV-2 (e.g., Sub201), unloading the rep and cap genes therein, maintaining only the ITRs on the both ends, then assembling sequentially the promotor, therapeutic genes, and poly A singal, which is to be improved for complexed steps and high cost.

The present invention simplifies the steps for constructing a AAV vector plamid carrying the therapeutic genes by providing a series of universal AAV vector plasmids and their construction method, as well as the methods for constructing a AAV vector plamid carrying the therapeutic genes by using said universal AAV vector plasmids.

The present invention relates to the construction of a series of universal AAV vector plasmids, which include pWAV-1, pWAV-2, pSNAV-1 and pSNAV-2. The common character thereof lies in that each vector provides ITRs from both ends of type 2 AAV, cytomegalovirus (CMV) immediate early enhancer and promoter, polyclonal sites, and polyA singal. The present invention provides a method to construct AAV vectors carrying exogenous genes using said universal AAV vectors. AAV vectors

carrying exogenous genes can be used not only for the production of recombinant AAVs, but also directly as eukaryotic expression plasmid. Besides, each of pSNAV-1 and pSNAV-2 additionally comprises a neomycin resistant gene cassette. Accordingly, the present invention provides a method for establishing a cell strain that carries stably the AAV vectors by using the exogenous gene-containing pSNAV-1 or pSNAV-2. The present invention further provides a method for production of recombinant AAVs via "one vector cell/one hepler virus", that is to say, infecting AAV vector cell strain using a full-function hepler virus invented earlier by us (CN Appl. No. 98120033.8) to realize a large-scale production of recombinant AAVs.

The original biological materials used in the invention are:

pSub201: a plasmid presented by Samulski laboratory containing the whole AAV-2 genome;

pUCMA, a plasmid made prviously by the present laboratorym, comprising CMV immediate early enhancer and promoter, polyclonal sites, and polyA;

pCMV/HyTK, an eukaryotic expression plasmid comprising CMV immediate early enhancer and promoter, and a HyTK gene under the control thereof;

pAV53, a AAV vector plamid carrying ampicillin resistant gene and E. coli replication origin;

pSV2neo, an eukaryotic expression plasmid manufactured by Promega expressing neomycin resistant gene.

pCMV-lacZ, an eukaryotic expression plasmid made prviously by the present laboratorym, comprising CMV immediate early enhancer and promoter, and a β-galactosidase gene under the control thereof;

pCD2, a retrovirus vector plasmid comprising CMV immediate early enhancer and promoter, and an E. coli cytosine deaminase (CD) gene under the control thereof, as well as a neomycin resistant gene under the control of the SV40 early promoter.

The host bacterium for the above plasmids is Escherichia coli MAX EFFICIENCY DH5α (GIBCO #18528-012)

The construction and characters of the series of universal AAV vector plasmids

The construction and characters of pWAV-1

See fig. 1. Rep and cap genes from pSub201 were cut by Xba I to leave the backbone of the plasmid and the ITRs on both ends of AAV-2 genome. And the CMV immediate early enhancer and promoter, polyclonal sites, and polyA sequences were unloaded from pUCMA by XbaI, then loaded between the above two ITRs, resulting the recombinant AAV vector plasmid pWAV-1.

This vector comprises ITRs from both ends of AAV-2, between the two ITRs sequentially are CMV immediate early enhancer and promoter, chimeric intron, polyclonal sites, and polyA singal. Wherein, the CMV immediate early enhancer and promoter, together with the chimeric intron are 1.1kb long. Said chimeric intron is consisted of the 5' splicer donor sites of the first intron from human β-globin, and the 3' splicer receptor sites of the variable region of heavy chain of immunoglobulin. Said chimeric

intron is placed upstream of the genes to be inserted, with the aim to splice mRNA precursor into mature mRNA at this site, as well as to prevent any possible splicing by a potential 5' splicer donor sites internal of the genes inserted. The polyclonal sites of said vector include such enzyme sites as Nhe I, Xho I, Pst I and BamH I, etc., which is convenient for the insertion of exogenous genes. The backbone of this plasmid is pEMBL, comprising the replication origin sequence necessary for replication in E. coli, as well as an ampicillin resistant gene. Since the maximal package limitation of rAAV is about 5kb, while said two ITRs, CMV immediate early enhancer and promoter, chimeric intron, polyclonal sites, and polyA singal are totally about 1.7kb, thus the length of exogenous genes that can be included is less than 3.3kb.

The construction and characters of pWAV-2

See fig. 2. HyTK gene was unloaded from pCMV/HyTK by double cut of Nhe I and Hind III, followed by recovery of the rest fragment of 2.9kb, with the sticky ends blunted by Klenow large fragment DNA polymerase and dNTP, then slef-ligated to constitute the recombinant pCMVPA. This pCMVPA was double cut by Xho I and BamH I to recover CMV and polyA fragment of 950bp. pAV53 was double cut by Xho I and BamH I to unload ampicillin resistant gene and E. coli replication origin while maintaining the backbone of the plasmid as well as the ITRs from both ends of AAV-2, which is ligated with the above CMV and polyA fragment to yield pWAV-2.

pWAV-2 also comprises ITRs from both ends of AAV-2, between the two ITRs sequentially are CMV immediate early enhancer and promoter, polyclonal sites, and polyA singal. The backbone of this plasmid contains the replication origin sequence necessary for replication in E. coli, as well as an ampicillin resistant gene. The polyclonal sites include Kpn I, EcoR I, Sal I, etc. The length of exogenous genes that can be included is less than 3.6kb.

The construction and characters of pSNAV-1

See fig. 3. Through a two-step cloning upon enzyme disgestion, blunting and re-ligation, the EcoR I and Bgl II sites were removed pSV2neo to yield pSV2neoΔEΔB. The LacZ expression cassette under the control of CMV promoter was cut from pCMV-lacZ by Xho I and BamH I, recovered, and then ligated into pAV53 between the ITRs resluted from double cut by Xho I and BamH I, forming the AAV vector plasmid pAV-LacZ carrying LacZ expression cassette. Said pAV-LacZ was cut by Bgl II to recover ITR-CMV-LacZ-ITR fragment, which was ligated at BamH I site into pSV2neoΔEΔB to form another AAV vector plasmid pSNAV-lacZ carrying LacZ expression cassette. Said pSNAV-lacZ was double digested by Xho I and BamH I to remove the LacZ expression cassette therein, which was replaced by CMV and polyA fragment obtained from pCMVPA upon double digestion of Xho I and BamH I, thereby resulted in the pSNAV-1.

pSNAV-1 comprises ITRs from both ends of AAV-2, between the two ITRs sequentially are CMV immediate early enhancer and promoter, polyclonal sites, and polyA singal. Besides, there is a neomycin resistant gene expression cassette under the control of SV40 promoter outside of the ITRs.

The backbone of this plasmid contains the replication origin sequence necessary for replication in E. coli, as well as an ampicillin resistant gene. The polyclonal sites include Kpn I, EcoR I, Sal I, Bgl II, etc. The length of exogenous genes that can be included is less than 3.6kb.

The construction and characters of pSNAV-2

See fig. 4. pCD2 was digested with Nhe I to unload CD-SV40-neo^r region, which was then inserted in forward direction into the Nhe I site of pWAV-1, thereby resulted in pWCDN. Said pWCDN was enzyme digested with BamH I to unload SV40-neo^r region, which was then assembled into the BamH I site of pWAV-2, thereby resulted in pSNAV-2.

Between the two ITRs on pSNAV-2, sequentially exists CMV immediate early enhancer and promoter, polyclonal sites, polyA singal, SV40 early promoter, a neomycin resistant gene expression cassette, and polyA singal. The backbone of this plasmid contains the replication origin sequence necessary for replication in E. coli, as well as an ampicillin resistant gene. The polyclonal sites include Kpn I, EcoR I, Sal I, etc. The length of exogenous genes that can be included is less than 1.9kb.

The above-mentioned four universal AAV vector plasmids are each preseved in Escherichia coli MAX EFFICIENCY DH5α (GIBCO #18528-012), which is subcultured at 37°C in LB medium containing 50-100 μg/ml ampicillin. The bacterium containing the plasmid pWAV-1 is named as DH5α/pWAV-1 (deposited under China General Mcirobiological Culture Collection Center with the accession number CGMCC No.0415.1). The bacterium containing the plasmid pWAV-2 is named as DH5α/pWAV-2 (deposited under China General Mcirobiological Culture Collection Center with the accession number CGMCC No.0415.2). The bacterium containing the plasmid pSNAV-1 is named as DH5α/pSNAV-1 (deposited under China General Mcirobiological Culture Collection Center with the accession number CGMCC No.0415.3). The bacterium containing the plasmid pSNAV-2 is named as DH5α/pSNAV-2 (deposited under China General Mcirobiological Culture Collection Center with the accession number CGMCC No.0415.4).

The usage of the series of universal AAV vector plasmids

For construction of recombinant AAV vector plasmids carrying exogenous genes

Depending on the size and the terminal enzyme restriction sites of the exogenous genes, selection can be made among pWAV-1, pWAV-2, pSNAV-1, or pSNAV-2 for loading. The specific procedure is to use restriction endonuclease corresponding to the polyclonal sites to cut the universal AAV vector plasmids, followed by ligation with exogenous genes treated by corresponding enzymes (e.g., T4 ligase), then using the resulted plasmid to transform competent E. coli. Upon screen of the recombinants, recombinant AAV vector plasmids carrying exogenous genes could be obtained.

For preparation of recombinant AAV carrying exogenous genes

Depending on the size and the terminal enzyme restriction sites of the exogenous genes, selection can be made among pWAV-1,

pWAV-2, pSNAV-1, or pSNAV-2 for loading. The pWAV-1, pWAV-2, pSNAV-1, or pSNAV-2 loaded with exogenous genes was co-transfected into cells together with a helper plasmid containing AAV-2 rep and cap genes using liposome (or calcium phosphate, or electroperforation), followed by infection with helper virus (such as adenovirus or herpes simplex virus), thereby a rAAV pseudovirus particle containing the exogenous genes could be packaged. Alternatively, using the full-function helper virus invented by us (CN Appl. No. 98120033.8) to infect cells having been transfected by pWAV-1, pWAV-2, pSNAV-1, or pSNAV-2 loaded with exogenous genes, a rAAV pseudovirus particle containing the exogenous genes could also be packaged.

pSNAV-1 or pSNAV-2 loaded with exogenous genes can be used to transfect cells using liposome or calcium phosphate, then screen cells on G418 pressure to give a G418 resistant cell, which is the recombinant AAV vector cell strain, this is because there is a neomycin resistant gene expression cassette in both pSNAV-1 and pSNAV-2. Using the full-function helper virus according to our invention to infect said cell strain, could package rAAV pseudovirus particles containing the exogenous genes.

For expression of exogenous genes in eukaryotic cells

Depending on the size and the terminal enzyme restriction sites of the exogenous genes, selection can be made among pWAV-1, pWAV-2, pSNAV-1, or pSNAV-2 for loading. Exogenous genes could be transiently expressed by transfecting mammal cells with pWAV-1, pWAV-2, pSNAV-1, or pSNAV-2 loaded with exogenous genes using liposome (or calcium phosphate, or electroperforation). pSNAV-1 or pSNAV-2 loaded with exogenous genes can be used to transfect mammal cells using liposome (or calcium phosphate, or electroperforation), then screen cells on G418 pressure to give a G418 resistant cell, which can enable the stable expression of exogenous genes.

EXAMPLES

The usages of the series of universal AAV vector plasmids are specified in the following examples, which should not be construed as limitations for the contents of the invention.

Example 1 Preparations of plasmid DNA

Plasmid DNA were extracted by the method of alkaline lysis according to Molecular Cloning –A Laboratory Manual, 2nd edition (Sambrook J. et al, 1986), and purified by the method of polyethylene glycol precipitation

Example 2 pWAV-1 is used to load E. coli cytosine deaminase (CD) gene

pCD2 was digested with Nhe I to unload CD-SV40-neo^r region of 2.8kb, separated by electrophoresis, then recovered and purified using glassmilk. pWAV-1 was digested with Nhe I for linearization, then it was ligated to CD-SV40-neo^r. Ligations were used to transform Escherichia coli MAX EFFICIENCY DH5α. The recombinants were identified by enzyme digestions, and a AAV vector plasmid pWCDN carrying E. coli cytosine

deaminase (CD) gene was screened.

Example 3 pSNAV-1 is used to load green fluorescence protein (GPF) gene

GFP was unloaded from pGreen Lantern-1 (purchased from GIBCO BRL) using Not I, and assembled into Not I site of pCDNA2.1 (purchased from INVITROGEH) to form recombinant plasmid pcDNA2.1/GFP(+/-).The recombinant plasmid which had the opposite orientation between GFP transcription and T7 promoter was named pcDNA2.0/GFP(-). The pcDNA2.0/GFP(-) was double digested by EcoR I and Xho I to recover the fragment GFP, which was inserted into the site between EcoR I and Sal I in pSNAV-1 to construct pSNAV–1-GFP carrying GFP. The plasmid comprised in turn the following elements: ITR-CMV-GFP-SV40 polyA-ITR-SV40 promoter-neo-polyA-amp^R-E.coli ori.

Example 4 pSNAV-2 is used to load green fluorescence protein (GPF) gene

The plasmid DNA PcDNA2.1A/GFP(-) was double cut by Kpn I and Xho I to recover the fragment GFP, which was inserted into the site between Kpn I and Sal I in pSNAV-2 to construct pSNAV–2/GFP. The plasmid comprised in turn the following elements: ITR-CMV-GFP-SV40 polyA-SV40 promoter-neo-polyA-ITR-amp^R-E.coli ori.

Example 5 the establishment of recombinant AAV vector cell strian carrying green fluorescence protein (GPF) gene

1.5 μg pSNAV–1-GFP and pSNAV–2-GFP were used individually to transfect 50% confluenced BHK-21 cells cultured in 6-well plate using 10 μl Lipofactamine (GIBCO BRL) according to the product description. Medium is changed after 24hrs, and cells were grown in 1640 medium (supplemented with 10% fetal bovine serum) containing 400 μg/ml G418. The resulting resistant cells after about 10 to 15 days are recombinant AAV vector cell strains carrying green fluorescence protein (GPF) gene.

Example 6 the production of recombinant AAV-GFP by cotransfection of plasmids

1.5 μg pSNAV–1-GFP (or pSNAV–2-GFP) was mixed with 3μg pAAV/Ad to transfect 80% confluenced BHK-21 cells cultured in 6-cm plate using 20 μl Lipofactamine (GIBCO BRL) according to the product description. Medium is changed every 5hrs, and cells were infected by type 5 adenovirus (MOI=2). Upon complete pathologic at 48-72hrs, the cells were disrupted for four times by freezing and thawing cycles, and cell debris were removed by low speed centrifugation, the supernatant was collected and subjected to heat at 56□ to inactivate the helper virus, then stored at -20□ until use.

Example 7 the production of recombinant AAV-GFP by infecting AAV-GFP vector cell strain using full-function helper virus

Full-function helper viruses at MOI of 0.1 were used to infect recombinant AAV vector cell strains carrying green fluorescence protein (GPF) gene established in Example 5. Upon complete pathologic at 24-48hrs, the cells and medium were subjected to four cycles of freezing and thawing, centrifuged at 100rpm/min for 5 min, the resulting

supernatant contained lots of rAAV-GFP viruses. Thus infectious rAAV-GFP virus could be conveniently produced by such a process, and batch production of rAAV could be realized.

Example 8 the transduction of cultured cells with recombinant AAV-GFP

1 ml rAAV-GFP virus supernatant was removed, and added into cultured BHK cells (80% confluence), lots of green cells could be observed under fluorescent microscopy (light excited is of 490 nm) after 24-48 hrs. It showed that rAAV thus produced is infectious, and capable of introducing exogenous genes into cells.

Example 9 the transient expression of GFP in cells

1.5 μg pSNAV–1-GFP (or pSNAV–2-GFP) was used to transfect 80% confluenced BHK-21 cells cultured in 6-cm plate using 20 μl Lipofactamine (GIBCO BRL), lots of green cells could be observed under fluorescent microscopy (light excited is of 490 nm) at 24 hrs. It showed that pSNAV–1-GFP (or pSNAV–2-GFP) can express GFP in eukaryotic cells, and that both pSNAV–1 and pSNAV–2 could be used as eukaryotic expression vectors.

Example10 the stable expression of GFP in cells

1.5 μg pSNAV–1-GFP and pSNAV–2-GFP were used individually to transfect 50% confluenced BHK-21 cells cultured in 6-well plate using 10 μl Lipofactamine (GIBCO BRL) according to the product description. Medium is changed after 24hrs, and cells were grown in 1640 medium (supplemented with 10% fetal bovine serum) containing 400 μg/ml G418. The resulting resistant cells after about 10 to 15 days are found to be green in color under fluorescent microscopy (light excited is of 490 nm), that is to say, GFP was expressed. GFP was continuously expressed with the passage of cells, which showed that both pSNAV–1 and pSNAV–2 could be used as eukaryotic expression vectors to mediate stable expressions of exogenous genes.

Figure 1 construction illustration of pWAV-1;
Figure 2 construction illustration of pWAV-2;
Figure 3 construction illustration of pSNAV-1;
Figure 4 construction illustration of pSNAV-2.

What we claimed is:

The present invention relates to the field of biotechnology, particularly to a series of universal adeno-associated virus (AAV) vector plasmids useful for gene transfer, gene therapy, and eukaryotic gene expressions.

1. Universal AAV vector plasmids pWAV-1 and pWAV-2 according to the present invention, characterized by comprising ITRs from both ends of type 2 AAV, between which ITRs sequentially are cytomegalovirus immediate early enhancer and promoter, polyclonal sites, and polyA singal.

2. Universal AAV vector plasmid pSNAV-1 according to the present invention, characterized by comprising ITRs from both ends of type 2 AAV, between which ITRs sequentially are cytomegalovirus immediate early enhancer and promoter, polyclonal sites, and polyA singal, as well as a neomycin resistant gene cassette outside the ITRs.

3. Universal AAV vector plasmid pSNAV-2 according to the present invention, characterized by comprising ITRs from both ends of type 2 AAV, between which ITRs sequentially are cytomegalovirus immediate early enhancer and promoter, polyclonal sites, polyA singal, and a neomycin resistant gene cassette.

4. Universal AAV vector plasmids according to the present invention used to carry exogenous genes, characterized by insertion of said exogenous genes into the polyclonal sites of said universal AAV vector plasmids.

5. Universal AAV vector plasmids according to the present invention used to produce recombinant AAVs carrying exogenous genes.

6. Universal AAV vector plasmids according to the present invention used to establish cell strains stably carrying recombinant AAVs, said cell strains are characterized by comprising, stably integrated into their chromosomes, AAV ITRs and exogenous gene expression unit.

7. Universal AAV vector plasmids according to the present invention used for eukaryotic cells for exogenous gene expression.

8. Universal AAV vector plasmids according to claim 1, 2 or 3, wherein a SV40 early enhancer and promoter locates in front of the polyclonal sites.

9. Universal AAV vector plasmids according to claim 1, 2 or 3, wherein the long terminal repeat region from genome of Rous sarcomas virus locates in front of the polyclonal sites.

10. Universal AAV vector plasmids according to claim 1, 2 or 3, wherein the thydimine kinase promoter from herpes simplex virus locates in front of the polyclonal sites.

11. Universal AAV vector plasmids according to claim 2 or 3, wherein the selective marker gene is hygromycin resistant gene.

12. Universal AAV vector plasmids according to claim 2 or 3, wherein the selective marker gene is dihydrofolate reductase gene.

13. Universal AAV vector plasmids according to claim 2 or 3, wherein the selective marker gene is xanthine-guanine phosphoribosyl transferase gene.

14. Universal AAV vector plasmids according to claim 2 or 3, wherein the selective marker gene is adenosine deaminase gene.

ABSTRACTS

The present invention relates to the construction of a series of universal adeno-associated virus (AAV) vectors, which include pWAV-1, pWAV-2, pSNAV-1 and pSNAV-2. The common character thereof lies in that each vector provides ITRs from both ends of type 2 AAV, cytomegalovirus (CMV) immediate early enhancer and promoter, polyclonal sites, and polyA singal. The present invention provides a method to construct AAV vectors carrying exogenous genes using said universal AAV vectors. AAV vectors carrying exogenous genes can be used not only for the production of recombinant AAVs, but also directly as eukaryotic expression plasmid. Besides, each of pSNAV-1 and pSNAV-2 additionally comprises a neomycin resistant gene cassette. Accordingly, the present invention provides a method for establishing a cell strain that carries stably the AAV vectors by using the exogenous gene-containing pSNAV-1 or pSNAV-2. The present invention further provides a method for production of recombinant AAVs via "one vector cell/one hepler virus", that is to say, infecting AAV vector cell strain using a full-function hepler virus invented earlier by us (CN Appl. No. 98120033.8) to realize a large-scale production of recombinant AAVs.

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Biochemistry

Cloning and expression of cDNA for rat heme oxygenase

(expression cDNA library/antibody screening/transient expression/enzyme induction/hemin)

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ABSTRACT **Two cDNA clones for rat heme oxygenase have been isolated from a rat spleen cDNA library in λgt11 by immunological screening using a specific polyclonal antibody. One of these clones has an insert of 1530 nucleotides that contains the entire protein-coding region. To confirm that the isolated cDNA encodes heme oxygenase, we transfected monkey kidney cells (COS-7) with the cDNA carried in a simian virus 40 vector. The heme oxygenase was highly expressed in endoplasmic reticulum of transfected cells. The nucleotide sequence of the cloned cDNA was determined and the primary structure of heme oxygenase was deduced. Heme oxygenase is composed of 289 amino acids and has one hydrophobic segment at its carboxyl terminus, which is probably important for the insertion of heme oxygenase into endoplasmic reticulum. The cloned cDNA was used to analyze the induction of heme oxygenase in rat liver by treatment with $CoCl_2$ or with hemin. RNA blot analysis showed that both $CoCl_2$ and hemin increased the amount of hybridizable mRNA, suggesting that these substances may act at the transcriptional level to increase the amount of heme oxygenase.**

The microsomal heme oxygenase plays an essential role in physiological heme catabolism (1, 2). Heme oxygenase catalyzes the oxidative degradation of heme to biliverdin (1), which is subsequently converted to bilirubin by biliverdin reductase (3). In the rat, the activity of heme oxygenase is highest in the spleen, where senescent erythrocytes are sequestrated and destroyed (2). Other tissues such as bone marrow and liver also perform this function, especially in hemolytic states and after splenectomy (2, 4). Heme oxygenase is highly inducible by its substrate heme in kidney (2, 5), liver (2, 5), and macrophages (6–8). Heme oxygenase is also induced by various other substances such as metal ions (9, 10), endotoxin (11), and bromobenzene (12).

We are particularly interested in the induction of heme oxygenase by heme, because heme (ferrous protoporphyrin IX) is an essential component of hemoglobin and of other hemoproteins. Furthermore, hemin (ferric chloride protoporphyrin IX) has interesting biological properties such as stimulation of neurite outgrowth (13), promotion of adipocyte differentiation (14), and stimulation of globin mRNA accumulation in erythroleukemic cells (15, 16). Previously, we demonstrated that hemin increased the levels of functional mRNA for heme oxygenase in cultured pig alveolar macrophages (17) and in rat liver (18), suggesting that hemin acts at the transcriptional level to increase the amount of heme oxygenase. To understand the molecular mechanisms of induction of heme oxygenase, it is essential to know the structure of the gene for heme oxygenase.

In this study, we have isolated cDNA clones for rat heme oxygenase by antibody screening, and we have confirmed that our cDNA actually encodes heme oxygenase by expressing cDNA in monkey kidney cells. We determined the nucleotide sequence of the cloned cDNA and deduced the amino acid sequence of heme oxygenase.

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MATERIALS AND METHODS

Preparation of RNA. Total RNA was prepared from rat spleen by the method of Chirgwin *et al.* (19). Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (20).

Construction of cDNA Expression Library and Antibody Screening. cDNA was synthesized from 10 μg of rat spleen poly(A)$^+$ RNA and converted to double-stranded cDNA by using RNase H and DNA polymerase I (21). Double-stranded cDNA was methylated at internal *Eco*RI sites with *Eco*RI methylase (22). Addition of *Eco*RI linkers and ligation to λgt11 DNA (23, 24) was carried out as described by Schwarzbauer *et al.* (25). After *in vitro* packaging (Amersham), the recombinant phage were plated on *Escherichia coli* strain Y1090 (24, 25) and incubated at 37°C for 6 hr with 2 mM isopropyl β-D-thiogalactopyranoside. Each plate was then overlaid with a nitrocellulose filter and incubated for 12 hr at 37°C. The filters were air dried, washed with 50 mM Tris·HCl, pH 7.5/150 mM NaCl (TBS) and treated with TBS containing 3% bovine serum albumin for 1 hr at room temperature. The following procedures were performed at room temperature unless otherwise indicated. The filters were washed with TBS containing 0.1% Triton X-100 (TBST) and treated with rabbit anti-rat heme oxygenase IgG (18) for 2 hr in TBST containing 3% bovine serum albumin. For reducing the background, anti-rat heme oxygenase IgG was treated with bacterial extracts at 4°C overnight, and any precipitate was removed by centrifugation. Then, the filters were washed extensively with TBST and treated with ^{125}I-labeled protein A (Amersham) in TBST containing 3% bovine serum albumin for 1 hr. Filters were washed with TBST, air dried, and autoradiographed overnight at −70°C with intensifying screens.

Subcloning and Sequencing of cDNAs. The *Eco*RI inserts of positive phage clones (λRHO2 and λRHO6) were purified, ligated into *Eco*RI-cleaved pUC8 plasmid, and used to transform *E. coli* HB101. To detect the fusion protein in bacterial colonies by antibody, *E. coli* DH-1 was used for transformation (26). The subcloned DNA fragments were used for further analysis, and nucleotide sequences were determined by the method of Maxam and Gilbert (27).

Cloning of Full-Length cDNA for Heme Oxygenase. A cDNA library was constructed by the method of Okayama and Berg (28) using 2.0 μg of rat spleen poly(A)$^+$ RNA and screened with ^{32}P-labeled *Dde* I(93)/*Dde* I(240) fragment as a hybridization probe. The numbers in parentheses, shown together with restriction enzymes, indicate the 5′-terminal nucleotide generated by cleavage (see Fig. 1). All DNA

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probes used in this study were labeled with [α-^{32}P]dCTP by the method of Feinberg and Vogelstein (29).

Construction of Expression Plasmids Carrying Heme Oxygenase cDNA. The expression vector pKCRH2 (30) was linearized by digestion with *Hin*dIII and single-stranded ends were filled in by treatment with DNA polymerase I (Klenow fragment, Boehringer Mannheim). The resulting blunt ends were used for ligation with the following inserts: (*i*) *Xho* I(−59)/*Hin*dIII(971) fragment isolated from pRHO1; (*ii*) *Eco*RI(88)/*Eco*RI(1429) fragment derived from λRHO6. Both ends of each fragment were converted to blunt ends before ligation. Recombinant plasmids were cloned in *E. coli* HB101 and plasmids for each insert with opposite orientation were isolated. The pKCRHO21 thus obtained carries the *Xho* I(−59)/*Hin*dIII(971) fragment in the same orientation with respect to simian virus 40 early gene transcription. pKCRHO21-anti contains the same insert as pKCRHO21 but in the opposite orientation. pKCRHO7 has the *Eco*RI(88)/*Eco*RI(1429) fragment in the same orientation with respect to simian virus 40 early gene transcription.

Transfection of COS-7 Cells and Assay of Heme Oxygenase. COS-7 cells (31) were maintained in Dulbecco's minimal essential medium containing 2.5% fetal calf serum and 2.5% newborn calf serum (32). Confluent cells, seeded in 140-mm dishes, were fed with medium 4 hr before addition of plasmid DNA. Plasmid DNA (60 μg per dish) was used to transfect COS-7 cells by the calcium phosphate method (33, 34). After a 48-hr incubation, cells were collected and stored at −70°C until assay for heme oxygenase. Thawed cells were disrupted by sonication and pellets (microsomes) were prepared by centrifugation at 105,000 × *g* (8). The microsomes were suspended in 50 mM potassium phosphate buffer, pH 7.4/0.1% Triton X-100, and assayed for heme oxygenase (35). One unit of the enzyme was defined as the amount catalyzing the formation of 1 nmol of bilirubin per min. Protein amount was determined by using commercial reagent (Bio-Rad).

RNA Blot Analysis. Total RNA was prepared from untreated rat liver and from liver treated with hemin or with $CoCl_2$. Administration of these reagents to rats was carried out as described (18). Total RNA (10 μg) was denatured (36), electrophoresed on a 1.1% agarose gel containing 1 M formaldehyde, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled *Eco*RI fragment (nucleotide residues −23 to 87) derived from λRHO2. The size markers were rat rRNA and *E. coli* rRNA (37).

RESULTS AND DISCUSSION

cDNA Cloning. Heme oxygenase represents ≈0.33% of total peptides synthesized on free polysomes isolated from pig spleen (38). Therefore, rat spleen poly(A)$^+$ RNA was used to construct cDNA libraries. From ≈8 × 10^5 cDNA clones in a λgt11 expression library, two phage clones, λRHO2 and λRHO6, were isolated by antibody screening (Fig. 1). *Eco*RI sites at both ends of λRHO6 were produced by the addition of *Eco*RI linkers. The 3′ *Eco*RI site was produced in the poly(dA) tract located 5 residues downstream from nucleotide residue 1429 (see Fig. 3). Thus, the clone λRHO6 contains 1530 nucleotides, except for linker sequences, as well as 5 residues of poly(dA) tract. For convenience, we indicate the 5′ and 3′ ends of λRHO6 as *Eco*RI(−101) and *Eco*RI(1429), respectively. Clone λRHO2 contains the insert of 110 nucleotides (nucleotide residues −23 to 87) except for a part of linker sequence at its 5′ end. The 5′ end of λRHO2 is indicated as *Eco*RI(−23). Apparently λRHO2 is derived from the cDNA in which the internal *Eco*RI site was not protected by *Eco*RI methylase against *Eco*RI digestion. For further analysis, three *Eco*RI fragments excised from both phage clones were subcloned in pUC8 plasmid.

Because λRHO6 is not a full-length cDNA in comparison with the estimated size of mRNA (≈1800 nucleotides) by RNA blot analysis (see Fig. 5), we constructed another cDNA library by the method of Okayama and Berg (28), and

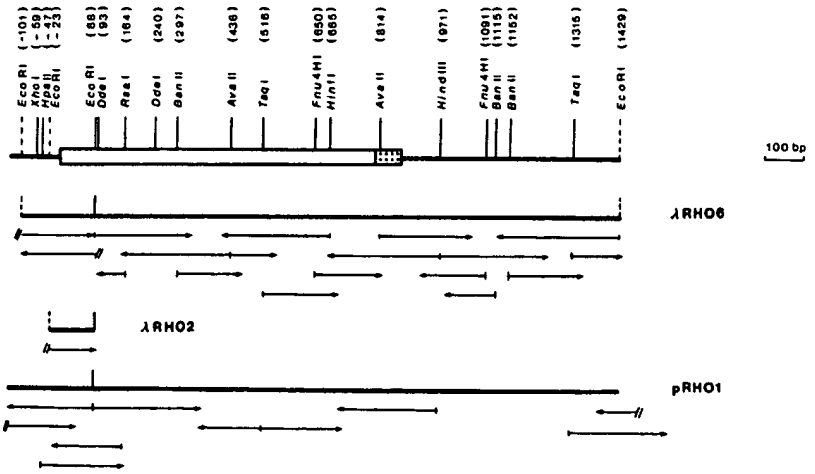

FIG. 1. Restriction map and sequencing strategy of cloned cDNA encoding rat heme oxygenase. Restriction map shows only the relevant sites, identified by numbers indicating the 5′-terminal nucleotide generated by cleavage (see Fig. 3). The only exception to this numbering is in the *Eco*RI sites produced by the addition of *Eco*RI linkers, indicated as dotted lines. The poly(dA) tract and poly(dG) tail are not included in the restriction map. The protein-coding region is indicated by an open box, and the putative membrane segment is indicated by a stippled box. The arrows indicate the direction and extent of sequence determinations. The short vertical lines and the slash marks at the end of arrows indicate the site of 5′-end labeling located in cDNA and vector DNA, respectively. The short solid and dotted lines on clones indicate the internal *Eco*RI sites and artificial *Eco*RI sites, respectively. bp, Base pairs.

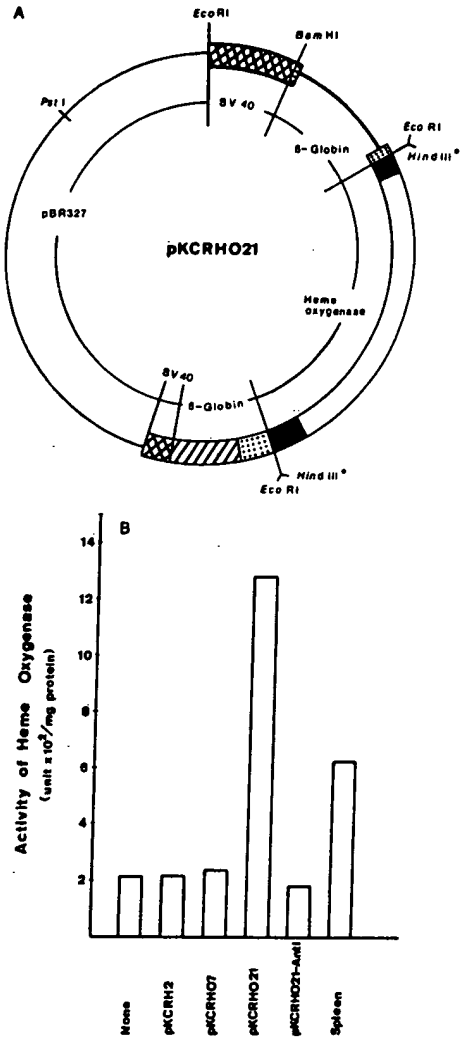

FIG. 2. Structure of expression plasmid carrying cDNA for heme oxygenase and expression of heme oxygenase in transfected COS-7 cells. (*A*) Schematic representation of expression plasmid pKCRHO21. The origin of the segments is shown on the inner circle. The protein-coding region of heme oxygenase cDNA is indicated by an open box and its 5′ and 3′ noncoding regions are shown by closed boxes. pKCRHO2 is represented the same way as in the original paper (30). Cross-hatched boxes, simian virus 40 (SV40) sequences; stippled boxes, exonic sequences of rabbit β-globin gene; solid heavy line, intronic sequence of rabbit β-globin gene; slashed box, its 3′ flanking sequence. The segment derived from plasmid pBR327 is indicated by solid line. Transcription occurs clockwise. *Hin*dIII sites, marked with closed circles, were converted to blunt ends and used to construct pKCRHO21 as well as pKCRHO21-anti and pKCRHO7. (*B*) Expression of heme oxygenase in transfected COS-7 cells. After transfection with the indicated plasmid, microsomal fractions were prepared for assay of heme oxygenase. Basal activity was measured in untransfected cells (indicated as none). For comparison, activity of rat spleen was included.

we screened the library with the *Dde* I(93)/*Dde* I(240) fragment as a hybridization probe. From ≈3 × 10⁵ transformants, we isolated pRHO1, which contains 1557 nucleotides excluding the dG tail and the poly(dA) tail (≈150 residues) (Fig. 1).

Expression of Heme Oxygenase cDNA in COS-7 Cells. As the amino acid sequence of heme oxygenase is not known, we sought to confirm that the isolated cDNA actually encodes heme oxygenase. Consequently, we transfected COS-7 cells with the expression vector pKCRH2 (31) containing our cDNA, and then we measured the activity of heme oxygenase. COS-7 cells have endogenous heme oxygenase activity and this basal activity did not change after transfection with plasmid pKCRH2 (Fig. 2*B*). Accordingly, we used the activity of heme oxygenase in cells transfected with pKCRH2 as a control.

The structure of the expression plasmid pKCRHO21 is indicated in Fig. 2*A*. The pKCRHO7, carrying the *Eco*RI-(88)/*Eco*RI(1429) fragment, lacks the amino-terminal segment of heme oxygenase (amino acid residues 1–29) (Fig. 3). The cells transfected with pKCRHO7 have the same activity of heme oxygenase as the control value (Fig. 2*B*). In contrast,

```
                                                    5'- - - - AC   -127
AGTCGCCAGTCGCCTCCAGAGTTTCCGCCTCCAACCAGCGAGTGGAGCCTGCCGGAGCAG   - 67
AGCCATCTCGAGCGGAGCCCGGAGCCTGAAGCCGAGCGGAGCCAGCCTGAACTAGCCCAGT  - 7
                    -13
CCCGCGATGGAGCGCCCACAGCTCGACAGCATGTCCCAGGATTTGTCCGAGGCCTTGAAG    54
      MetGluArgProGlnLeuAspSerMetSerGlnAspLeuSerGluAlaLeuLys
       1                  10
GAGGCCACCAAGGAGGTGCACATCCGTGCAGAGAATTCTGAGTTCATGAGGAACTTTCAG   114
GluAlaThrLysGluValHisIleArgAlaGluAsnSerGluPheMetArgAsnPheGln
   20                  30
AAGGGTCAGGTGTCCAGGGAAGGCTTTAAGCTGGTGATGGCCTCCTTGTACCATATCTAT   174
LysGlyGlnValSerArgGluGlyPheLysLeuValMetAlaSerLeuTyrHisIleTyr
   40                  50
ACGGCCCTGGAAGAGGAGATAGAGCGAAACAAGCAGAACCCAGTCTATGCCCCGCTCTAC   234
ThrAlaLeuGluGluGluIleGluArgAsnLysGlnAsnProValTyrAlaProLeuTyr
   60                  70
TTCCCTGAGGAGCTGCACCGAAGGGCTGCCCTAGAGCAGGACATGGCCTTCTGGTATGGG   294
PheProGluGluLeuHisArgArgAlaAlaLeuGluGlnAspMetAlaPheTrpTyrGly
   80                  90
CCCCACTGGCAGGAGGCCATCCCTTACACACCAGCCACACAGCACTACGTAAAGCGTCTC   354
ProHisTrpGlnGluAlaIleProTyrThrProAlaThrGlnHisTyrValLysArgLeu
  100                 110
CACGAGGTGGGAGCTACTCATCCTGAGCTGCTGGTCGCCCACGCATATACCCGCTACCTG   414
HisGluValGlyGlyThrHisProGluLeuLeuValAlaHisAlaTyrThrArgTyrLeu
  120                 130
GGTGACCTCTCAGGGGGTCAGGTCCTGAAGAAGATTGCGCAGAAGGCCATGGCCTTGCCA   474
GlyAspLeuSerGlyGlnValLeuLysLysIleAlaGlnLysAlaMetAlaLeuPro
  140                 150
AGCTCTGGGGAAGGCCTGGCTTTTTCACCTTCCCGAGCATCGACAACCCCACCAAGTTC   534
SerSerGlyGluGlyLeuAlaPhePheThrPheProSerIleAspAsnProThrLysPhe
  160                 170
AAACAGCTCTATCGTGCTCGCATGAACACTCTGGAGATGACCCCCGAGGTCAAGCACAGG   594
LysGlnLeuTyrArgAlaArgMetAsnThrLeuGluMetThrProGluValLysHisArg
  180                 190
GTGACAGAAGAGGCTAAGACCGCCTTCCTGCTCAACATTGAGCTGTTTCAGGAGCTGCAG   654
ValThrGluGluAlaLysThrAlaPheLeuLeuAsnIleGluLeuPheGluGluLeuGln
  200                 210
GCACTGCTGACAGAGGAACACAAAGACCAGAGTCCCTCACAGACAGAGTTTCTTCGCCAG   714
AlaLeuLeuThrGluGluHisLysAspGlnSerProSerGlnThrGluPheLeuArgGln
  220                 230
AGGCCTGCTAGCCTGGTTCAAGATACTACCTCTGCAGAGACGCCCCGAGGAAAATCCCAG   774
ArgProAlaSerLeuValGlnAspThrThrSerAlaGluThrProArgGlyLysSerGln
  240                 250
ATCAGCACTAGTTCATCCCAGACACCGCTCCTGCGATGGGTCCTCACACTCAGTTTCCTG   834
IleSerThrSerSerGlnThrProLeuLeuArgTrpValLeuThrLeuSerPheLeu
  260                 270
TTGCCGACCGTGGCAGTGGGAATTTATGCCATGTAAATGCAGTGTTGGCCCCCAGAGGCT  894
LeuAlaThrValAlaValGlyIleTyrAlaMetEnd
  280                 289
GTGAACTCTGTCTCATGTAGCCTTCTCTCTGCAGGGGAGAATCTTGCCTGGCTCTCTTTT  954
CTTGGCCTCTAAGAAAGCTTTTGGGGTTCCTCGCCCCCTTCCTGTGTCTTCCTTTGTCT  1014
CTCTGGAATGGAAGGAGATGCCTGGCACATTTCCCTCACCAAAAGCACAGCCAGGGCCT  1074
GAACTTGGAAACCAGCAGCCCCAAATCCTGCAACAGAGCCCCAGAACTGTGTCGTCGTAGA  1134
GGCGGCTGTTCTGAGCCCAGTGCCCTTGGTAGTCGGCGTCCATGTTGACTGACCATGACT  1194
GCTTTCCCCCAGCGTCATGGCCACTTTGATATCAGTGTGCAGAGATTTTCTGTCTTGTGT  1254
TTCTGTCTTGTTTTTGTTATTTCCCCAGTTCTACCAGTGTAATGGTATTTTTTGTTGTAT  1314
CGATTGTTTTTTTATTCTAACCAAGTAGGGCTGTCTTTTGAGGGGTGGGTGGTATTTAAT  1374
AATTGTAACCTTGGTCTCTAACTTCTGTGTGAAATAATAAATGGCATTATCTAAT- - 3' 1429
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FIG. 3. Nucleotide sequence of cDNA for rat heme oxygenase and its deduced amino acid sequence. The nucleotide sequence of the message strand is shown. Nucleotides are numbered in the 5′ to 3′ direction and numbers are shown on the right side of the sequence. Nucleotide residue 1 is the A of the initiating methionine codon ATG, and the nucleotides on the 5′ side of residue 1 are indicated by negative numbers. The deduced amino acids are shown below the nucleotide sequence and are numbered beginning with the initiating methionine. The putative membrane segment and the polyadenylylation signal, AATAAA (39), are underlined. The poly(dA) tract (≈150 residues) is not included.

DECLARATION

I, Xiaobing Wu, a citizen of China, residing at No. 6 Yongchang Zhonglu; BDA Beijing 100176; PEOPLE'S REPUBLIC OF CHINA; and

I, Tung Yu Tsui, a citizen of China, residing at L9-55, Faculty of Medicine Building, 21 Sasson Road, Pokfualm; Hong Kong; PEOPLE'S REPUBLIC OF CHINA;

hereby state that we are the inventors of US Patent Application No. 10/750,620, filed on December 30, 2003 and entitled:

"METHOD TO PREVENT TRANSPLANT REJECTION BY STABLE EXPRESSION OF HEME OXYGENASE-1"

and that we are the authors of the publication *Circulation* 2003, 107: 2623-2629 who conceived of the above invention,

whereas the other persons, i.e., Chi-Keung Lau, David W.Y. Ho, Tao Xu, Yeung-Tung Siu and Sheung-Tat Fan, who were listed in the above publication as authors, actually did not participate in this conception, their contribution to the invention were merely in providing experimental support.

We declare under penalty of perjury under the laws of the United States of America, and under penalty of the laws of any other jurisdiction before which this document may be presented, that we have signed this document as our own free act and that all of the foregoing is true and correct.

Dated: Mar. 30. 2006

Xiaobing Wu

Xiaobing Wu, Inventor

Dated: Mar. 30. 2006

Tung Yu Tsui

Tung Yu Tsui, Inventor